25pp

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Steroidogenesis:
Detailed Review Paper

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Overview

- Considerations in the Selection of a Screening Approach
- In Vitro Approaches to Evaluating Steroidogenesis
  - Strengths / Limitations
- Use of Tissues vs. Primary Cell Preparations
- Candidate Steroidogenesis Protocol
  - Review of Strengths / Limitations as a Screening Approach
- Cell Lines
- Recommendations
- Candidate Chemicals

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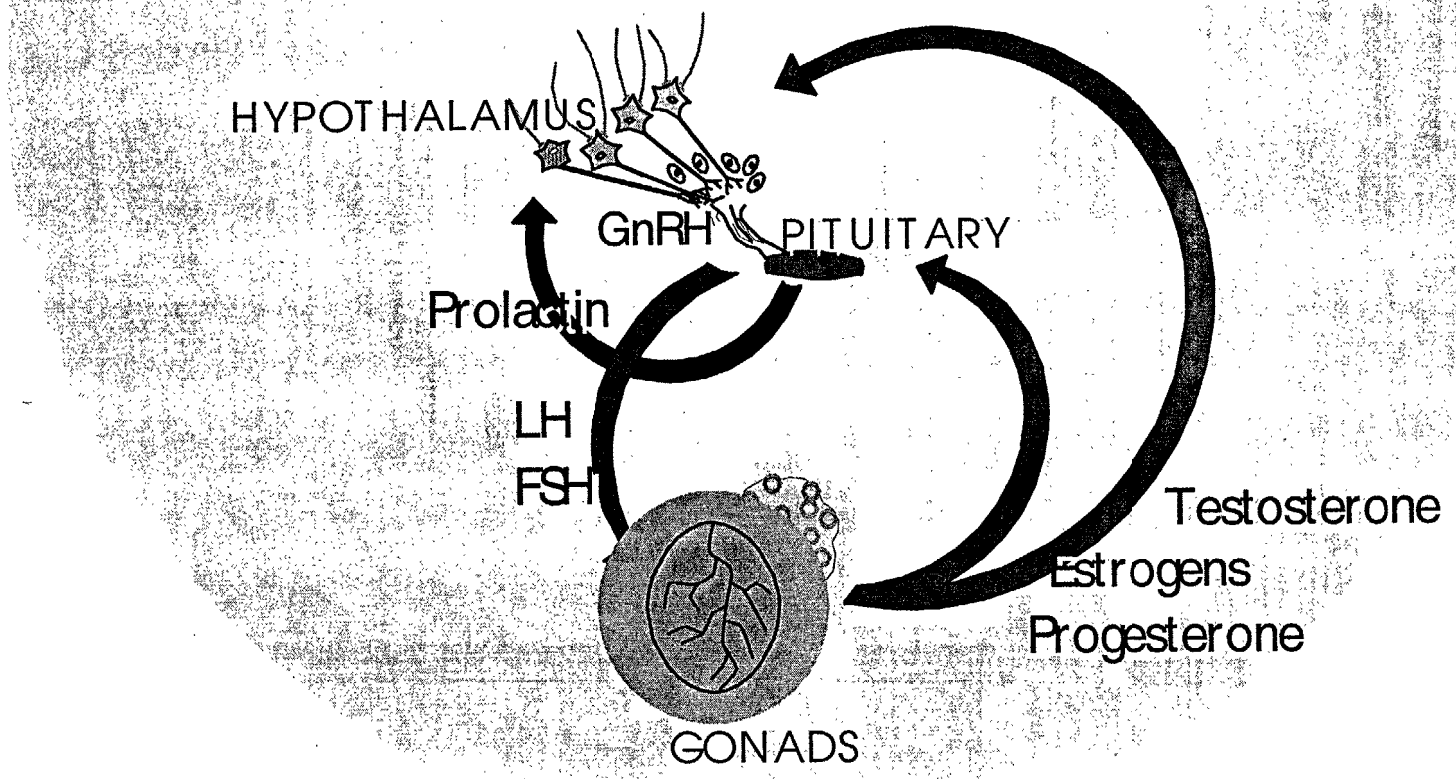
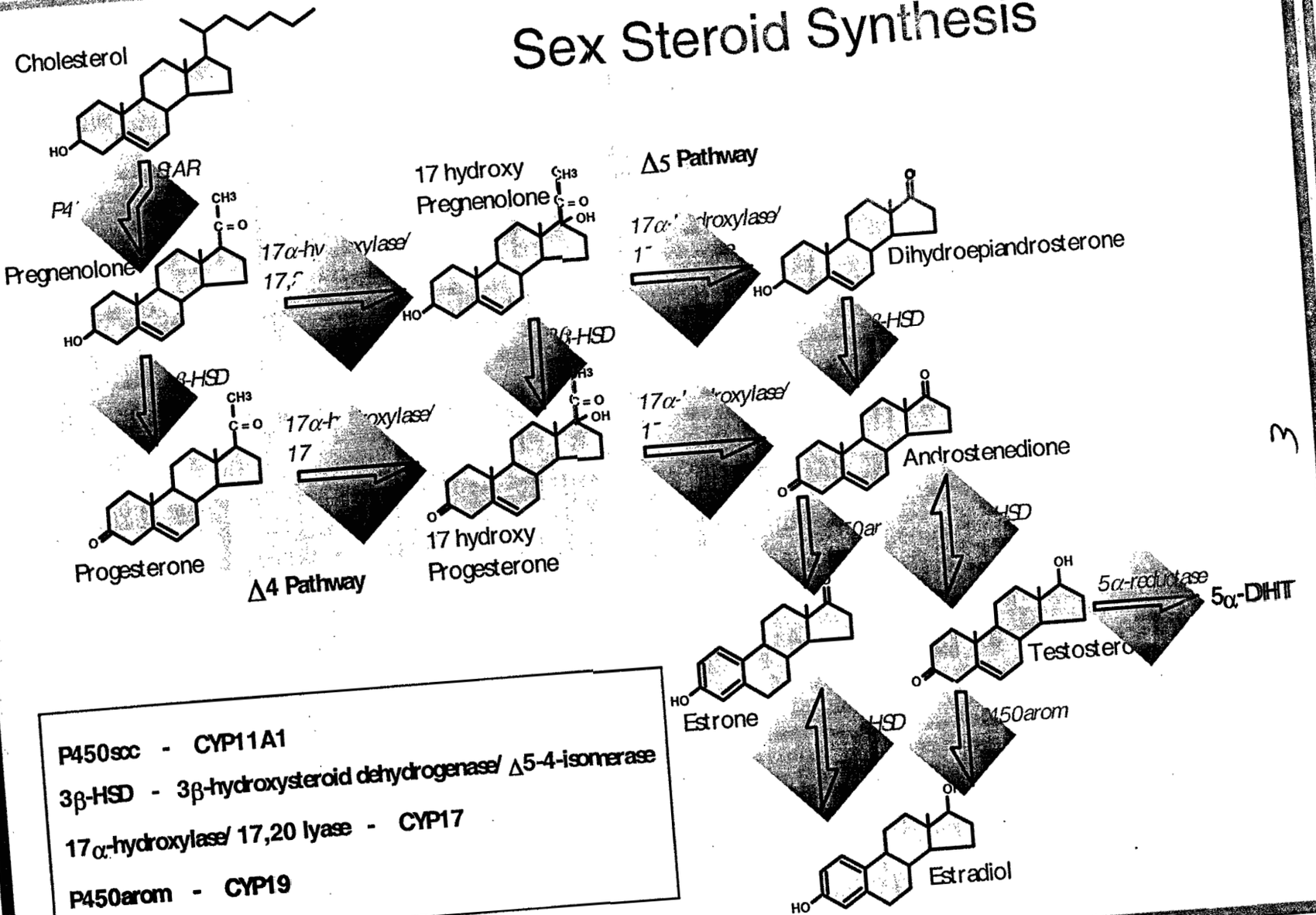
Hypothalamic-Pituitary-Gonadal Axis



Sex Steroid Synthesis



P450scc - CYP11A1

3β-HSD - 3β-hydroxysteroid dehydrogenase/ Δ5-4-isomerase

17α-hydroxylase/ 17,20 lyase - CYP17

P450arom - CYP19

Selection of an Screening Approach to Evaluate a Toxicant Effect on Steroidogenesis: Considerations

- Predictiveness
- Sensitivity
- Variability (Intra- & Inter-laboratory)
- Animal Use (Refine / Reduce / Replace)
- Ease of Use
- Standardization
- Cost (Personnel / Equipment)
- Time Requirements (Personnel Time / Stability of Prep)
- Multiple Samples Evaluated (Throughput)
- Metabolic Activation

Steroid Evaluation Using In Vitro Approaches

Gender

- Male
- Female

Exposure

- In vitro
- Ex vivo

Biological Material

- Tissues
- Organs
- Cells: Primary culture / Established cell line

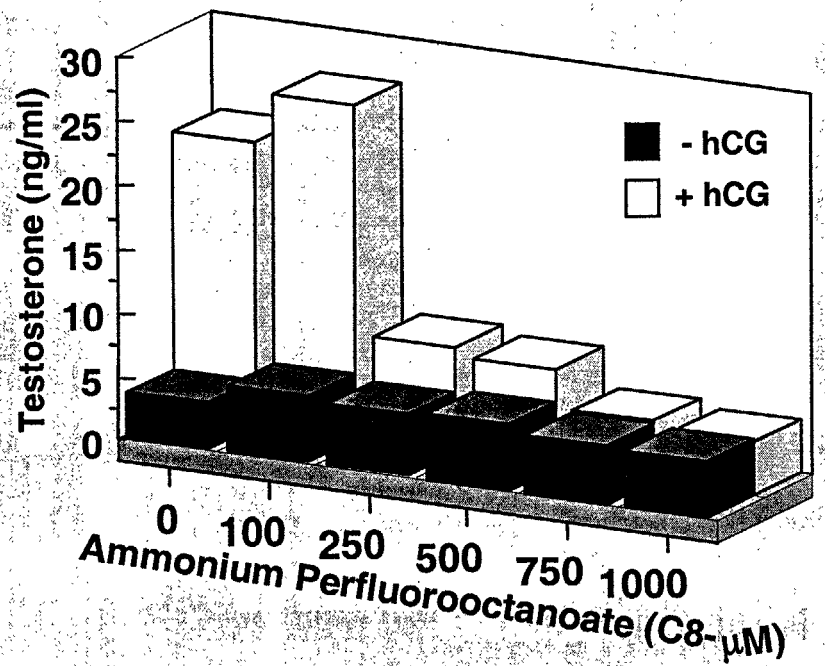
Sampling

- Incubation Vial
  - Single Sample
  - Multiple Samples: Media Replacement or Cumulative Sampling
- Flow-through Approach
  - Perifusion
  - Perfusion

Approach	Strengths	Limitations
In Vitro	-Exposure limited to tissues/ cells of interest- specificity of response -Random assignment of tissues/ cells to treatments reduces variability -Reduction in animals use -Shorter exposure times / higher throughput -Less material needed -Lower costs	-Lack of metabolic activation -Issues of general toxicity of compound in vitro -Solubility of the compound in culture -If cell cultures employed, maintenance can add an additional level of complexity -Sophisticated equipment may be required -Positive response in vitro, but failure to reach target tissue in vivo
In Vivo	-Standard routes of exposure -Systemic exposures allow for metabolism / normal interactions among organs &/or tissues. -Allows for more extended periods of exposure	-Increased costs / animal usage -Indirect effects on steroidogenesis hypothalamic-pituitary effects, changes in body weight, systemic toxicity

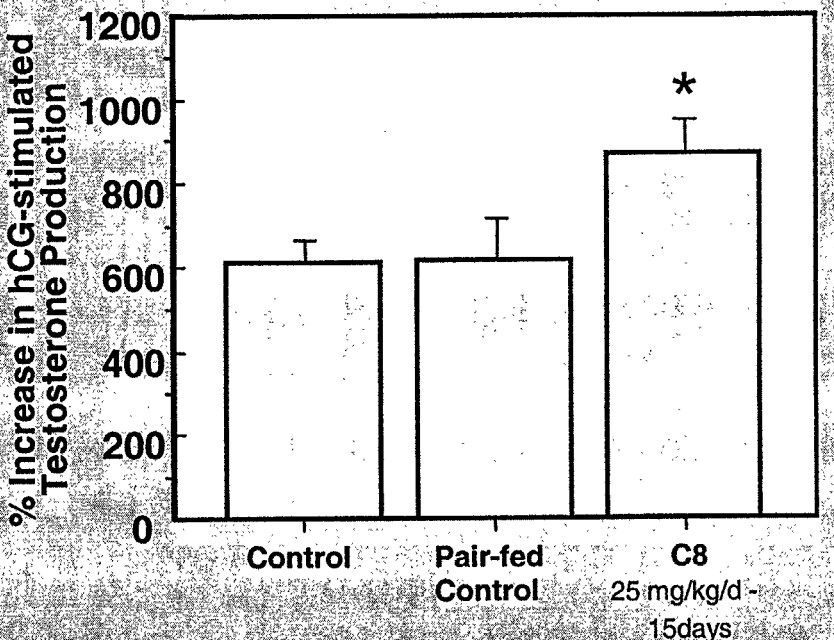
Type of Exposure	Strengths	Limitations
In Vitro In vitro exposure In vitro sampling	-Exposure limited to tissues/cells of interest -Random assignment of tissues/cells to treatment conditions reduces variability -Reduction in number of animals required / shorter exposure times	-Maintenance of cell cultures can add an additional level of complexity -Added level of concern about general toxicity of compound in vitro -Solubility of the compound in culture
Ex Vivo In vivo exposure In vitro sampling	-Allows for more extended periods of exposure -Systemic exposures allow for normal interactions among organs &/or tissues. -Standard routes of exposure	-More limited control of exposure levels compared to in vitro approach -Movement of compound out from the cells/tissues in culture may alter the response characteristics present in vivo

Effects of In Vitro Exposure to Ammonium Perfluorooctanoate[1] on Testosterone Production



[1]Derivative of perfluorocarboxylic acid plasticizers

Ex Vivo Testosterone Production from Isolated Rat Leydig cells in Response to Ammonium Perfluorooctanoate



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Data from Biegel et al. Toxicol. Appl. Pharmacol. 134:18-25, 1995. Presented as Steroidogenesis DRP- Fi[illegible]4-

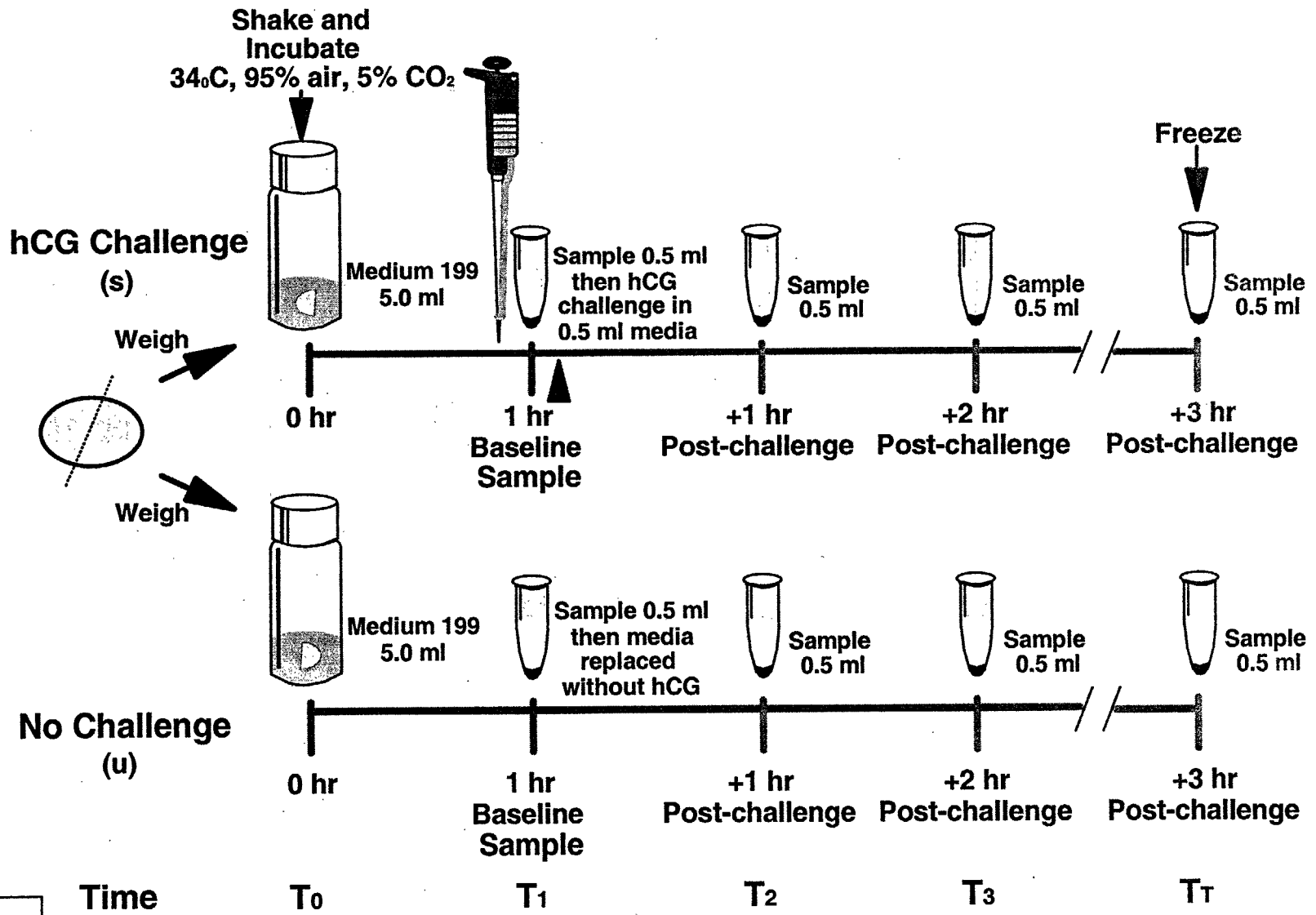
Steroidogenesis DRP: In Vitro Approaches Reviewed

- Isolated Organs (Perfusion / Perifusion)
  - Testis / Ovary
- Sectioned / Minced Tissue
  - Testis / Ovary
- Primary Cell Preparations
  - Leydig cells / Granulosa cells
- Cell Lines

Comparison Summary of In Vitro Methods – Table 4-11

Parameter	Whole Testis (simple incubation)	Perfused Testis	Perifused Testis	Sectioned Testis	Isolated & Cultured Leydig Cells (crude)	Isolated & Cultured Leydig Cells (purified)	Cell Lines
Cost	$	$$$	$$$	$	$$	$$$	$
Time: - Initial Set-Up	Day(s)	Week(s)	Week(s)	Day(s)	Day(s)	Week(s)	Week(s)
- Conduct	~30 to 50 testes/day (estimate)	~2 testes/day (deduced; Koos et al., 1984)	~ 2 testes/day (deduced; Koos et al., 1984)	~30 to 50 testes/day (deduced; EDSTAC, 1998)	~12 testes/day (deduced; Klinefelter et al., 1993)	~12 testes/day (Klinefelter et al., 1993)	Un-determined
Lab: - Training	General	Specialized	Specialized	General	General	Specialized	Specialized
- Equipment	General +	Specialized + + +	Specialized + + +	General +	General + +	Specialized + + +	Specialized + +
Standardization (Level of Difficulty)							
Animal Usage	♂♂♂	♂♂♂	♂♂♂	♂♂	♂	♂	**None**
Cytoarchitecture	Intact organ	Intact organ	Intact organ	Semi-intact organ	Incomplete organ (with cellular debris)	Incomplete organ	**Transformed / Un-differentiated cell**
Stability (Viability)	6 hours (+) (Deb et al., 1980)	4.5 hours (+) (Chubb / Ewing, 1979b)	no data	5 hours (+) (Laskey et al., 1994)	4-6 hours (Biegel et al., 1995)	48 hours (+) (Thoreux-Manlay et al., 1995)	3 hours (+) (Chaudhary / Stocco 1989)
Sensitivity	no data	15 Inhibitors @ 30 μM - inhibited T from 1 to 95 % (Chubb / Ewing, 1979b)	no data	Detect $_{\Delta}$ @ μM conc. (Laskey et al., 1994)	Detect $_{\Delta}$ @ μM conc (Laskey and Phelps, 1991)	Detect $_{\Delta}$ @ μM conc (Kelce et al, 1991	Detect $_{\Delta}$ @ μM conc (Chaudhary / Stocco, 1989)
Metabolic Activation	None	None	None	None	None	Add an S9 fraction (evidence is equivocal)	Add an S9 fraction (evidence is equivocal)
Endpoints	Enzyme act. (Deb et al., 1980	Steroid hormones (11) (Chubb & Ewing, 1979b)	Steroid hormones (deduced)	Steroid hormones (5) (Gurler & Donatsch, 1979)	Steroid hormones (5) (Bambino & Hsueh 1981)	Steroid hormones (4) Enzyme Act. Histology (Kelce et al., 1991; Biegel et al., 1995; Klinefelter et al., 1991)	Steroid hormones (2) (Hoelscher and Ascoli, 1996)
Specificity	+ +	+ +	+ +	+ +	+ +	+ +	+ (+)

Sliced Testis Assay



Steroidogenesis DRP- Figure 5-1

Minced Tissue Steroidogenesis Assay

Strengths

- Not Difficult to perform
- Tissue quickly obtained & readied for incubation. Less personnel time involved.
- In vitro or ex vivo exposures.
  - Ex vivo approach allows for any metabolic activation to occur.
- Use of non-stimulated or stimulated conditions under varying concentrations of compound.

Limitations

- Use of whole minced tissue increases variability.
- For ovarian tissue, female cycling status can affect results. Steroid release from variable numbers of preovulatory follicles, corpora lutea.
- Need to eliminate early traumatic hormonal release when obtaining baseline values (Correctable)
- Use of animals- can vary depending on ex vivo or in vitro exposure designs

Biological Material	Strengths	Limitations
Cells	-Uniform cell type can be employed that may well reduce interassay variability & increase magnitude of response -Characterized cell line could reduce interlab variability -Improved penetration of compound	-Maintenance of cell cultures can add an additional level of complexity -Discrepancies among cell lines in ease of maintenance & characteristics of steroid secretion -If cells isolated from toxicant-exposed animals, will increase assay time considerably -Loss of tissue structural integrity
Tissues	-Maintenance of architectural integrity / interaction among different cell types -For ex vivo exposure, less time to removal of tissue & placement in medium than for isolated cells	-In vitro penetration of compound into tissue will vary, depending on nature and size of tissue -Compared to isolated cells or cell lines, less uniformity of test samples can add to variability

Assessment of Cell / Tissue Viability

Cells

- Dye Exclusion (trypan blue)
- Tetrazolium Dye Based Assays (e.g., MTT reduction)
- ATP Bioluminescence Assay

Tissues

- Lactic Dehydrogenase
- ATP Bioluminescence Assay
- Cytokine Release

Control Group Coefficients of Variation: Testosterone Secretion

Preparation	Non-stim.	LH/hCG-stim.	Reference
Sliced Testis	23	-	Gray et al. (1995) TAP 130:248.
	28	29	Powlin et al. (1998) Tox. Sci. 46:61.
	23	22	Wilker et al. (1995) Toxicology 95:93.
	50	-	Banczerowski et al. (2001) Br.Res. 906:25
Crude Leydig Cell Prep (~12-15%)	45	30	Chambon et al. (1985) Andrologia 17:172.
	26	27	Kan et al. (1985) J. Steroid Biochem. 23: 1023
	40	25	Raji & Bolarinwa (1997) Life Sci. 61:1067.
	15	12	Laskey & Phelps (1991) TAP 108:296.
Purified Leydig Cell Prep (80-95%)	11	23	Ronco et al. (2001) Toxicology 159:99.
	9	-	Nagata et al. (1999) FEBS Lett. 444:160.
	6	8	Romanelli et al. (1997) Life Sci. 61:557.
	-	12	Klinefelter et al. (1991) TAP 107:460.
	13	12	Guillou et al. (1985) FEBS Lett. 184:6.

Incubation parameters: 10^5-10^6 cells/well; 3-4h collection period; 100mIU hCG or 50 ng/ml oLH stimulat

Isolated Cells: Considerations in Selection

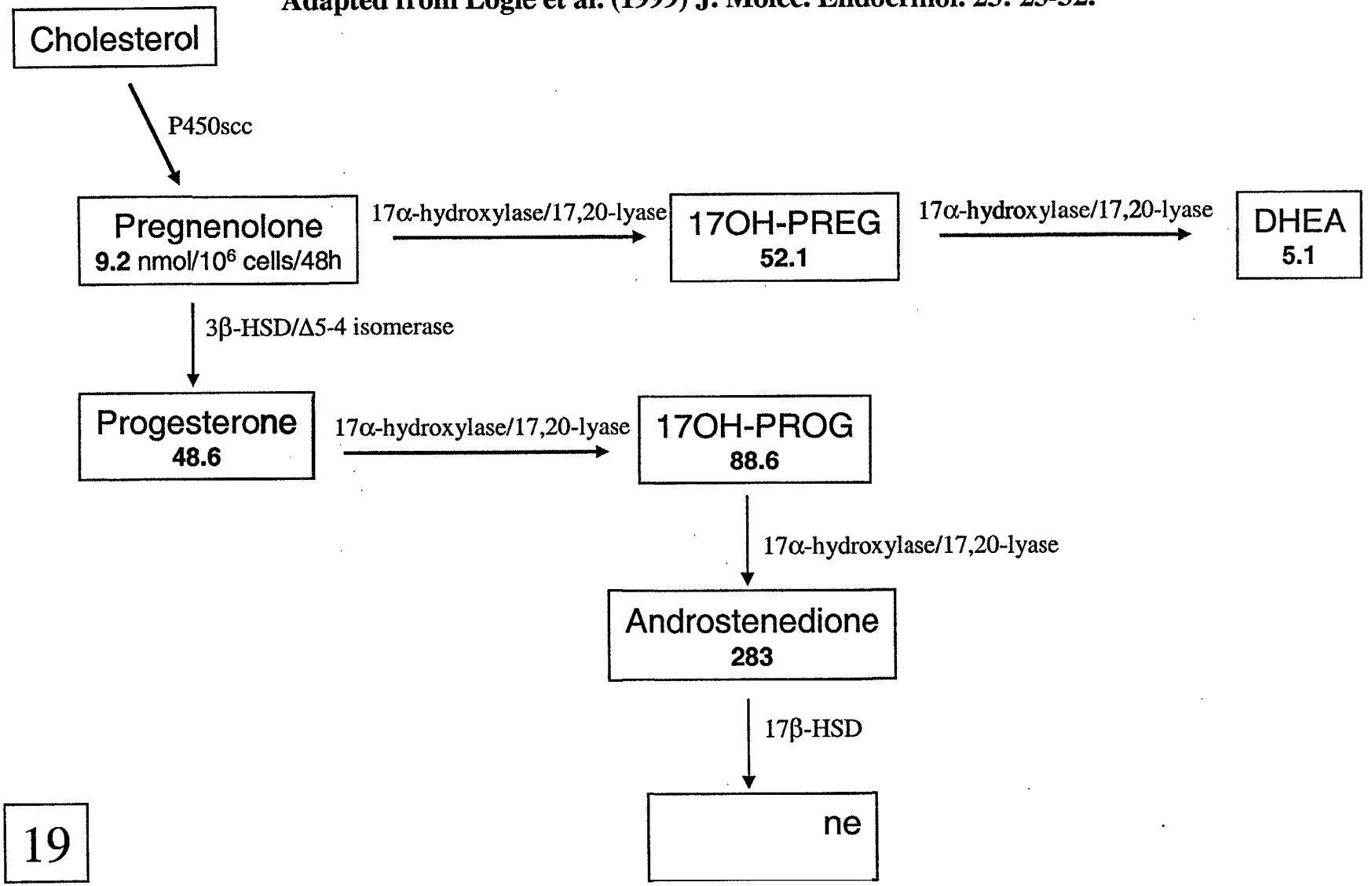
- Steroidogenically Active
- Endpoints of Interest / Appropriateness of Cell Type
- Primary Culture vs. Characterized Cell Line
- Assessment of Enhanced and Diminished Secretion
  - Non-stimulated vs. Stimulated Release
- Availability / Cost
- Ease of Maintenance

Examples of Cell Lines Employed in Studies of Steroidogenesis

		Comments
MA-10	**(mouse Leydig cell tumor line)**	Employed for pregnenolone / P4 production & StAR expression. Low basal P4; marked stimulated release. Very low T- recent report; induced by db-cAMP & hCG.
R2C	**(rat Leydig cell tumor line)**	High basal P4; limited stimulated release; high levels of P450arom & 5α-reductase.
H540	**(rat Leydig cell tumor line)**	Employed for evaluation of early steps in pathway (cholesterol ⇨ progesterone). Can produce androgens with db-cAMP pretreatment. Loss of responsiveness to hCG/LH.
mLTC-1	**(mouse Leydig cell tumor line)**	P4 & T. Loss of receptors under hCG.
H295R	**(human adrenocortical cell tumor line)**	Aromatase evaluations. High basal 3β-HSD; lower basal 17α-hydroxylase. Possibly useful to study entire pathway. Ease of maintenance?
KGN	**(human granulosa-like tumor cell line)**	Relatively high aromatase (stimulated by db-cAMP and FSH. P4 secretion responsive to db-cAMP stimulation. Minimal (if any) baseline secretion of DHEA, androstenedione or estradiol (17α-hydroxylation appears absent).
HO-23	**(immortalized human granulosa cell line)**	P4 secretion.
Jc-410	**(stable porcine granulosa cell line)**	Primarily P4, some E2 measurements; loss of responsiveness to gonadotropins. .

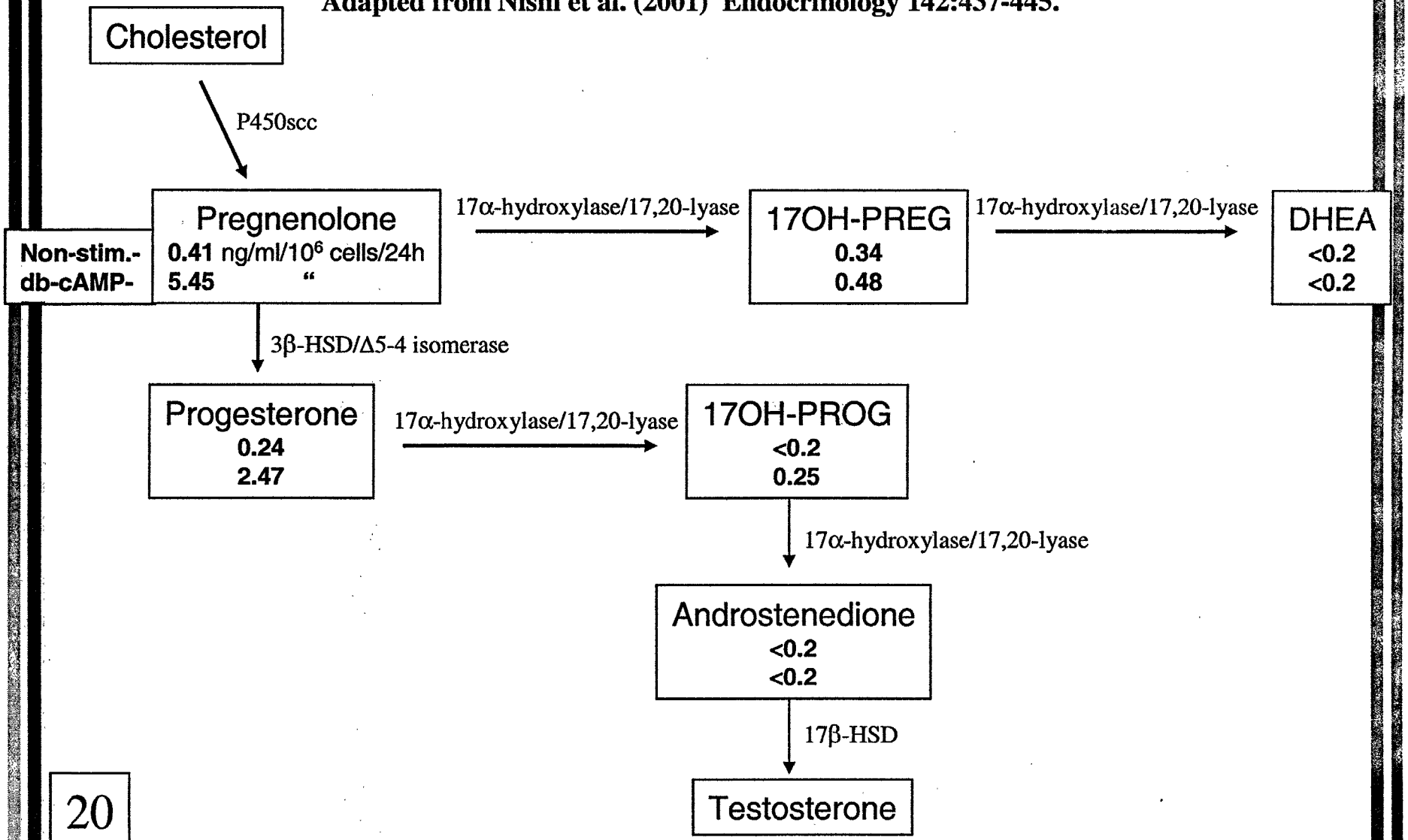
Steroid Production by H295R Cells

Adapted from Logié et al. (1999) J. Molec. Endocrinol. 23: 23-32.



Steroid Production by KGN Cells

Adapted from Nishi et al. (2001) Endocrinology 142:437-445.



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Recommendations

DRP Recommendation

- **Sliced Testis** (*Quartered, in vitro exposure*) - **Testosterone**

  -Advantages: Ease of use, reduced preparation / personnel time, reduced animal use, female cyclicity not an issue.

  -Limitations: Variability, lower sensitivity compared to purified cell preps.

- **Inclusion of assessments of tissue viability.**

Alternative

- **Explore feasibility of using a cell line as an alternative**

  -H295R – possibility that entire steroidogenic pathway (including aromatase activity) can be evaluated. [ATCC availability]

  -MA-10 – Commonly employed for progesterone release, so good database available. [M. Ascoli, Univ. Iowa]

Candidate Chemicals for Prevalidation

Ketoconazole (Mixed P450 inhibitor)

Cyanoketone (3β-HSD inhibitor)

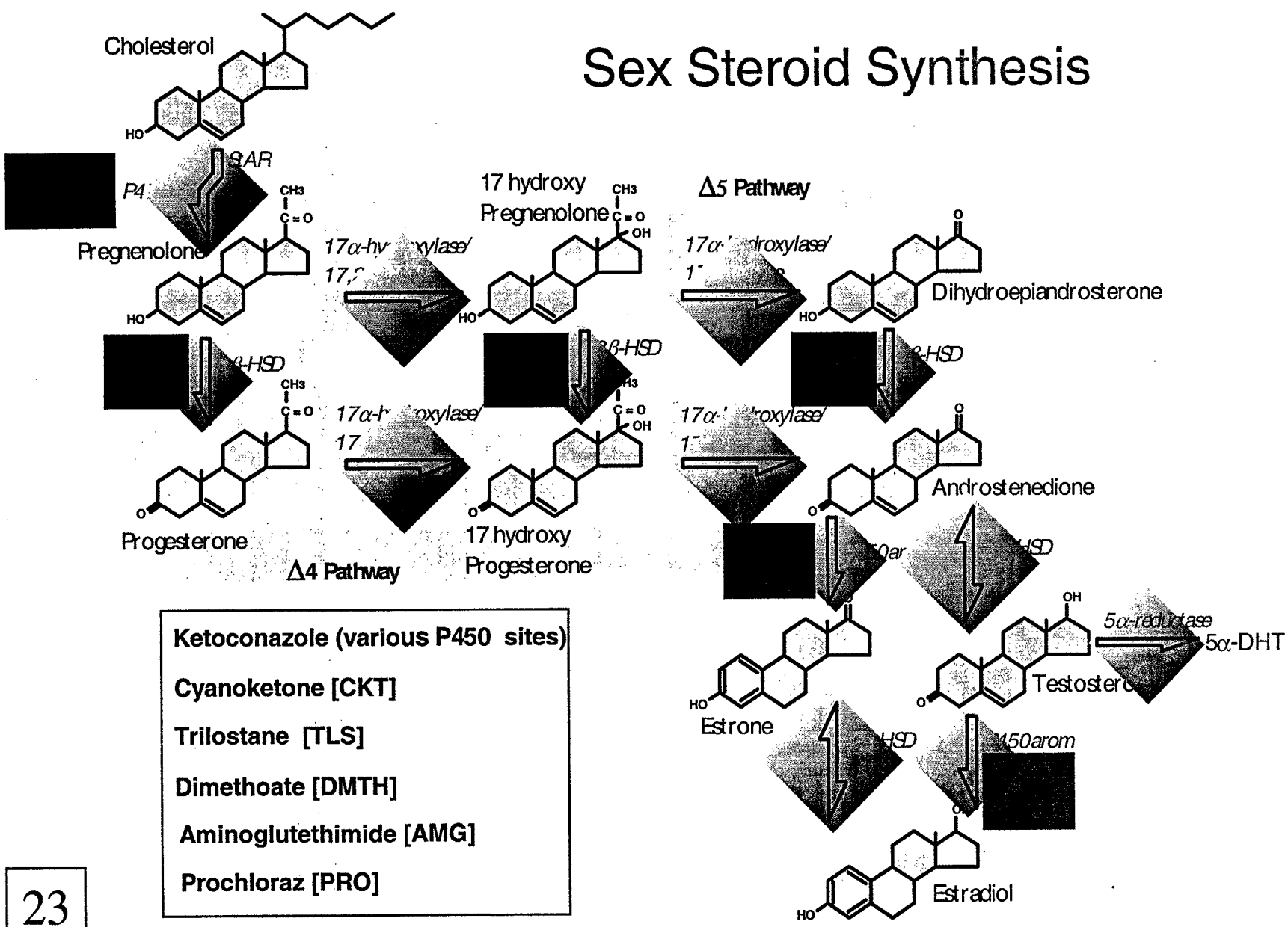
Trilostane (3β-HSD inhibitor)

Dimethoate (pesticide; Inhibits StAR expression / Suppression of cholesterol side-chain cleavage)

Aminoglutethimide (Aromatase inhibitor / Cholesterol side-chain cleavage inhibitor)

Prochloraz (fungicide; Aromatase inhibitor)

Sex Steroid Synthesis



- **Ketoconazole (various P450 sites)**
- **Cyanoketone [CKT]**
- **Trilostane [TLS]**
- **Dimethoate [DMTH]**
- **Aminoglutethimide [AMG]**
- **Prochloraz [PRO]**

Appendices

Selected Examples of Hormonal Actions

A1

Actions of Testosterone: Selected Examples (Male)

- Differentiation of internal reproductive tract and external male genitalia during fetal development. Sexual differention of CNS.
- Maturation of internal reproductive tract and external genitalia at puberty
- Accessory sex gland function (with conversion to dihydro-testosterone)
- Stimulation of spermatogenesis
- Anabolic action, growth of long bones
- Regulation of gonadotropin secretion

A2

Actions of Progesterone: Selected Examples (Female)

- Together with estradiol, regulates cyclicity- feedback effects on GnRH, LH, FSH secretion.

- Maternal ovarian maintenance of pregnancy. Subsequent placental production.

- Secretion by corpus luteum:
  -preparation of uterine endometrium for possible pregnancy
  -inhibits new follicular development and uterine contractions during pregnancy

- Increases mammary gland alveolar-lobular formation

A3

Actions of Estradiol: Selected Examples (Female)

- Growth / maintenance of female reproductive tract. Pubertal development
- Increases granulosa cell proliferation.
- Increases growth of endometrium and myometrium.
- Increases progesterone receptors in endometrium.
- Regulation of LH surge / cyclicity.
- Increases development of secondary sex characteristics.
- Stimulates duct development in mammary tissue.
- Effects on behavior
- Functions as a neuroprotectant